# Targeted disruption of the mouse *cis*-retinol dehydrogenase gene: visual and nonvisual functions

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**Abstract** **It has been proposed that *cis*-retinol dehydrogenase (cRDH) acts within the body to catalyze the oxidation of 9-*cis*-retinol, an oxidative step needed for 9-*cis*-retinoic acid synthesis, the oxidation of 11-*cis*-retinol [an oxidative step needed for 11-*cis*-retinal (visual chromophore) synthesis], and 3α-hydroxysteroid transformations. To assess in vivo the physiological importance of each of these proposed actions of cRDH, we generated cRDH-deficient (cRDH$^{-/-}$) mice. The cRDH$^{-/-}$ mice reproduce normally and appear to be normal. However, the mutant mice do have a mild visual phenotype of impaired dark adaptation. This phenotype is evidenced by electroretinagram analysis of the mice and by biochemical measures of eye levels of retinoid intermediates during recovery from an intense photobleach. Although it is thought that cRDH is expressed in the eye almost solely in retinal pigment epithelial cells, we detected cRDH expression in other retinal cells, including ganglion cells, amacrine cells, horizontal cells, and the inner segments of the rod photoreceptor cells. Aside from the eye, there are no marked differences in retinoid levels in other tissues throughout the body for cRDH$^{-/-}$ compared with cRDH$^{+/+}$ mice. Moreover, we did not detect any nonvisual phenotypic changes for cRDH$^{-/-}$ mice, suggesting that these mice do not have problems in metabolizing 3α-hydroxysteroids. Thus, cRDH may act essentially in the visual cycle but is redundant for catalyzing 9-*cis*-retinoic acid formation and 3α-hydroxysteroid metabolism.**—Shang, E., K. Lai, A. I. Packer, J. Paik, W. S. Blaner, M. de Morais Vieira, P. Gouras, and D. J. Wolgemuth. **Targeted disruption of the mouse *cis*-retinol dehydrogenase gene: visual and nonvisual functions. *J. Lipid Res.* 2002.** 43: **590–597.**

**Supplementary key words** vitamin A • retinoid • vision • knockout mice • 11-*cis*-retinol • steroid • retinol • eye

In previous work, we reported the identification of an enzyme that is present in mammary tissue, testis, liver, and kidney and that catalyzes the oxidation of 9-*cis*-retinol to 9-*cis*-retinal (1, 2). We first termed this enzyme 9-*cis*-retinol dehydrogenase (1) and later *cis*-retinol dehydrogenase (cRDH) (2). This enzyme appears to be identical to 11-*cis*-retinol dehydrogenase first identified in the bovine retinal pigment epithelium (RPE) (3–5). Although originally described as an eye-specific enzyme that was proposed to be importantly involved in the visual cycle (3–6), it now appears that this enzyme is present in a variety of nonocular adult tissues (including the testis, kidney, liver, and mammary tissue) and embryos (1, 7, 8). Moreover, in addition to using 9-*cis*- and 11-*cis*-retinoids as substrates, recent studies indicate that in vitro 3α-hydroxysteroids may also serve as substrates for this enzyme (9, 10).

The broad tissue distribution of cRDH and its ability to catalyze metabolic transformations of members of both the retinoid and steroid hormone families makes this enzyme potentially a very potent mediator of physiologic responses within the body. For this reason, it is important to understand the normal physiological actions of cRDH in the living organism, within both ocular and nonocular contexts. We have disrupted the mouse gene for cRDH by gene targeting and now report results of our studies of these mutant mice regarding the potential role of this enzyme in both the eye and other tissues within the body. Since cRDH-deficient (cRDH$^{-/-}$) mice show a visual phenotype of impaired dark adaptation, cRDH does play a role in the visual cycle, albeit a nonessential one as the mutant mice are not blind. The cRDH$^{-/-}$ mice appear to be otherwise phenotypically normal, suggesting that cRDH does not play a unique role in catalyzing formation of 9-*cis*-retinoic acid and/or bioactive steroids within the living organism.

Abbreviations: zCRALBP, cellular retinaldehyde-binding protein; CRABP, cellular retinoic acid-binding protein; cRDH, *cis*-retinol dehydrogenase; cRDH$^{-/-}$ mice, *cis*-retinol dehydrogenase-deficient mice; cRDH$^{+/+}$ mice, wild type mice; RPE, retinal pigment epithelium; ERG, electroretinagram; RXR, retinoid X receptor; RALDH2, retinaldehyde dehydrogenase type 2.

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## MATERIALS AND METHODS

### Gene targeting

Overlapping λ clones encompassing the complete cRDH gene were isolated from a mouse 129SvEv genomic library, restriction mapped, and sequenced at exon-intron boundaries. A targeting vector was constructed with two homologous flanking fragments of 5 kb (*Nae*I-*Nae*I) and 3.7 kb (*Kpn*I-*Kpn*I), and exon 3 and most of exon 2 were deleted. Vector pPNT was used in the construction of the targeting vector (11). First the 5 kb *Nae*I fragment was subcloned into the *Xma*I site of pBlueScriptSKII. Then the fragment was removed with *Not*I and *Xho*I and inserted into the *Not*I and *Xho*I sites of pPNT. The 3.7 kb *Kpn*I fragment was directly inserted into the *Kpn*I site of pPNT between phosphoglycerate kinase (PGK)-*neo* and HSV-TK. The construct was linearized at the *Not*I site and electroporated into 129/Sv ES cells and screened for positive $neo^r$ colonies using G418. Selected clones were analyzed by Southern blot using a 5′ diagnostic probe. Targeted cell clones were expanded and injected into C57Bl/6J blastocysts as described (12).

### Northern hybridization analysis

Total RNA was isolated from tissues using standard procedures (13). Total RNA samples (10 μg) were electrophoresed on 0.8% agarose gels containing 2.2 M formaldehyde at 1 V/cm for approximately 16 h. Ethidium bromide staining of the 18S and 28S rRNAs was used to determine equal loading for each total RNA sample. After electrophoresis, the gels were soaked in 20× SSC for 1 h and blotted overnight onto a nitrocellulose membrane using 10× SSC, and baked at 80°C in a vacuum oven for 2 h. The cRNA probe was labeled using T7 RNA polymerase and [α-$^{32}$P] UTP using a full-length cRDH cDNA as template. Hybridization was carried out overnight at 65°C in 5× SSC, 20 mM sodium phosphate buffer (pH 7.0), 60% formamide, 1% SDS, 5× Denhardt's solution, 100 μg/ml salmon sperm DNA, 100 μg/ml yeast RNA, and 7% dextran sulfate. After hybridization, the final wash was at 80°C in 0.2× SSC and 0.1% SDS for 1 h.

### Electroretinagrams

Mice were dark-adapted overnight before each experiment and ensuing procedures were performed under dim red light. The mice were anesthetized with a mixture of 80 mg/kg ketamine (Ketaset, Fort Dodge Animal Health, Fort Dodge, IA) and 10 mg/kg xylazine (Rompun, Phoenix Pharmaceuticals St. Joseph, MO), administered intraperitoneally. The pupils were dilated with 1% phenylephrine-HCl (Mydfrin, Alcon Laboratories Inc. Fort Worth, TX) and 1% tropicamide (Mydriacil, Alcon Laboratories Inc.) eye drops. Each mouse was placed on a heated stage in order to maintain body temperature at 37°C. A saline-moistened cotton wick contacted the cornea as the recording electrode. A needle placed subcutaneously on the forehead served as the reference electrode. A similar ground electrode was placed subcutaneously on the trunk. The electroretinagram (ERG) responses were amplified and averaged by a computerized data acquisition system (PowerLab, AD Instruments, Mountain View, CA). The light stimulus was obtained from a stroboscope (Grass PS33, Grass Instruments Inc., Warwick, RI) placed 8 cm in front of the eye and subtended 20° of visual angle. We consider that most of the light stimulation is due to scattered light in addition to focal light on the retina. The intensity of stimulation was changed by the interposition of neutral density filters. In order to determine if there was retinal degeneration, intensity-amplitude functions were determined for 11 $cRDH^{+/+}$, 11 $cRDH^{+/-}$, and 11 $cRDH^{-/-}$ mice. In order to examine dark-adaptation of the retina, dark-adaptation of 4 $cRDH^{+/+}$ and 4 $cRDH^{-/-}$ mice were compared. Four $cRDH^{+/+}$ and four $cRDH^{-/-}$ mice were light adapted for 5 min by a 6 V tungsten filament covered by a diffuser and placed 5 cm from the pupil. The brightness of this adapting field was 200 cd/m$^2$. The ERG was recorded at 1 min after the adapting light was turned off and then every 2 min thereafter for 2 h. We used a-wave amplitude to track the recovery of retinal function because the a-wave is almost an exclusively rod function in the mouse.

### Bleaching and recovery procedures used in biochemical measurements

The mice were anesthetized and the pupils were dilated as above (for ERG analysis). The mice were placed in a light box containing fluorescent lights with diffusers on all four sides. The brightness of the inner lighting side of the light box was 800 cd/m$^2$ and that of the reflecting side was about 600 cd/m$^2$. After a 1 h bleach, the mice were placed in the dark for recovery and at specific times the mice were sacrificed and the eyes were removed in red light and immediately frozen in liquid nitrogen.

### Retinal extractions from whole mouse eyes

Whole mouse eyes were extracted using a reported method (14), with minor modification. Extractions were carried out under dim red light. Individual pairs of frozen whole mouse eyes were homogenized in a Dounce homogenizer in 2 ml of 50 mM MOPS, pH 6.5, 10 mM $NH_2OH$ (freshly neutralized with HCl), and 50% ethanol, and homogenized with seven passes of the pestle. Homogenates were then incubated at 25°C for 30 min to allow for formation of retinal oximes. All-trans-9-(4-methoxy-2,3,6-trimethyl phenyl)-3,7-dimethyl-2,4,6,8-nonatetraen-1-01 (TMMP-ROH, RO12-0586) was added as an internal standard to assess loss during extraction. Retinal oximes were extracted into 4 ml of hexane and analyzed by normal phase HPLC as described below.

### Retinoid analysis

For analysis of retinol and retinyl esters, individual pairs of frozen whole mouse eyes were homogenized in a Dounce homogenizer in 2 ml of PBS and the proteins were denatured through addition of 2 ml of 100% ethanol. Retinoids were then extracted into 5 ml of hexane. Retinyl esters were saponified using 50% KOH in methanol at 65°C for 3 h followed by extraction into hexane. Retinol isomers were analyzed by normal phase HPLC as described below.

Retinol and retinal oxime isomers were separated on a Supelcosil LC-Si column (4.6 × 150 mm, 3 μm, Supelco Inc., Bellfonte, CA) using a mobile phase consisting of hexane-ethyl acetate-butanol (96.9:3:0.1, v/v/v) at a flow rate of 0.8 ml/min for 60 min (1). Retinoids were detected by UV absorption on a Waters 996 photodiode array detector. Retinol isomers were detected at 325 nm and retinal oxime isomers at 350 nm.

Serum retinol and tissue total retinol (retinol + retinyl ester) concentrations were determined by reverse-phase HPLC using a procedure we have previously described (15). Briefly, to an aliquot of serum (or tissue homogenates) an equal volume of absolute ethanol containing a known amount of the internal standard retinyl acetate (Sigma Chemical Co., St. Louis, MO) was added. Endogenous retinol and retinyl esters and the internal standard were extracted into hexane. After one backwash with $H_2O$, the hexane extract was evaporated to dryness under a gentle stream of $N_2$. Immediately upon reaching dryness, the retinoid containing film was redissolved in 40 μl of benzene for injection onto the HPLC. Retinol and retinyl esters were analyzed on a 4.6 × 250 mm 5 μm Beckmann Ultrasphere $C_{18}$ column (Beckmann Instruments, Inc.). The mobile phase consisted of acetonitrile-methanol-dichloromethane (70:15:15, v/v/v) delivered at a flow rate of 1.8 ml/min. Retinoids were detected and quantitated by UV absorbance at 325 nm using a Waters 996 photodiode array detector.

### In situ hybridization analysis

Dissected eyes were fixed, processed, and embedded in paraffin following standard procedures (16). Non-radioactive (digoxigenin) in situ hybridization was carried out on 6 μm sections according to Komminoth (17). Preparation of the digoxigenin-labeled cRDH probes was carried out using the Roche DIG DNA Labeling and Detection Kit according to the manufacturer's instructions. In addition to the full-length cRDH probe, a shorter probe excluding sequences 5′ of a *Hin*dII site 477 nt downstream of the initiation codon was also used. This shorter probe does not contain any sequences present in the targeted cRDH allele, and thus serves to confirm a lack of cRDH expression in tissues from knockout mice. Hematoxylin staining of sections was by standard protocols. Sections were photographed on a Leitz Dialux 20 microscope using Fujicolor 100 film.

## RESULTS

### Targeted disruption of the cRDH gene and generation of cRDH$^{-/-}$ mice

The targeting strategy used in this study is illustrated in **Fig. 1**. We used the 5 kb *Nae*I fragment as the left arm, the 3.7 kb *Kpn*I fragment as the right arm, and deleted exon 3 and most of exon 2 (total about 1 kb), which covers 197 out of 319 amino acids of the cRDH open reading frame. The cRDH promoter region was left intact due to the presence of another transcription unit (GCN5L1) approximately 500 bp upstream (18). After screening about 400 G418-resistant clones by Southern hybridization, three correctly targeted clones were identified. Cells were injected into C57Bl/6J blastocysts, the embryos implanted, and 10 male chimeras were obtained. Chimeric mice were then bred with wild-type C57BL/6J females and germline transmission was confirmed by the agouti coat color of the offspring and by Southern hybridization. In a targeted allele, insertion of a *Bam*HI site and a (PGK)-*neo* cassette converts the wild-type 9 kb *Bam*HI fragment to an 8.5 kb fragment. Heterozygotes were interbred to generate homozygous cRDH$^{-/-}$ mice.

### Northern analysis of cRDH gene expression

Northern hybridization was carried out on tissues from wild-type, heterozygous, and homozygous mutant mice. High levels of cRDH expression were observed in the eyes of cRDH$^{+/+}$ mice with lower levels in kidney, liver, and testis. No cRDH transcripts (1.4 kb) were detected for any of these tissues from cRDH$^{-/-}$ mice (data not shown). However, we did detect a small (0.8 kb) transcript upon Northern analysis for eyes, kidney, and liver from cRDH$^{-/-}$ mice. This transcript may be an alternatively spliced mRNA that is produced because of the intact upstream cRDH promoter. Since nearly two-thirds of the open reading frame was deleted, however, it is unlikely that this small transcript represents a functional cRDH mRNA.

### cRDH-deficiency and retinoid-related parameters

The cRDH$^{-/-}$ mice appear to be phenotypically normal. If cRDH$^{+/-}$ mice are mated, the genotypes of progeny mice are observed at expected Mendelian frequen-

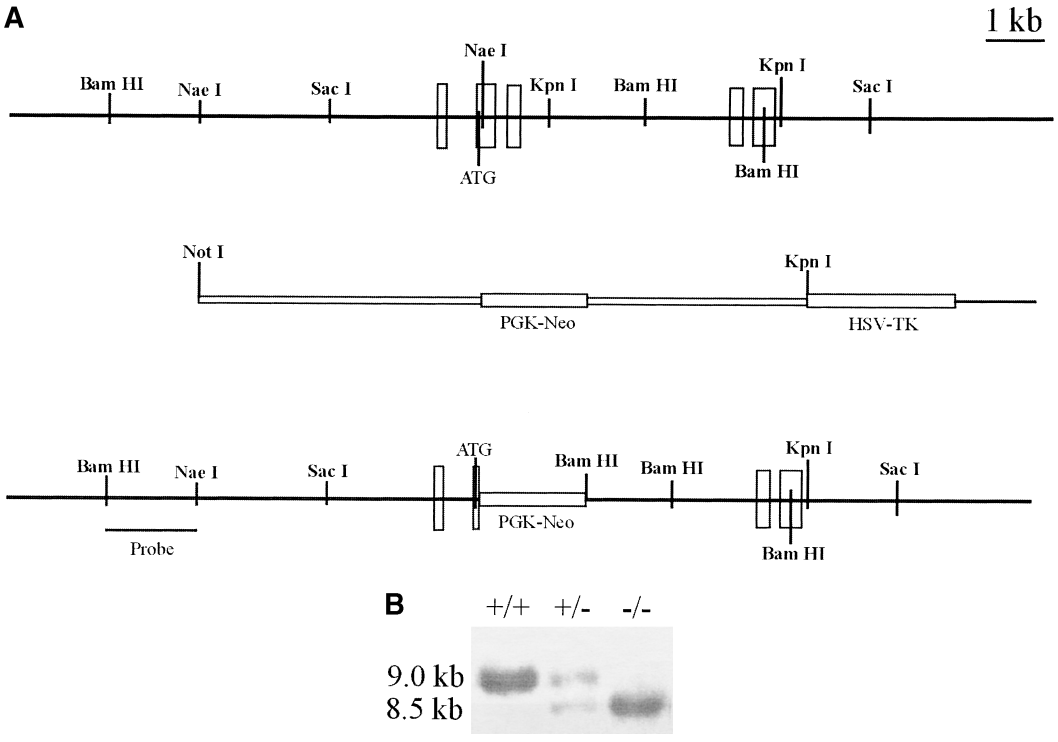


**Fig. 1**. Targeted disruption of the mouse *cis*-retinol dehydrogenase (cRDH) gene. A: Genomic organization of the cRDH locus and the structure of the deletion targeting vector. Exons are depicted as open boxes. In the mutant allele, a phosphoglycerate kinase (PGK)-*neo* cassette replaced 1 kb of the cRDH locus, encompassing exon 3 and most of exon 2 and resulting in deletion of sequences encoding 197 out of the 319 amino acids present in cRDH. B: Genomic DNA of littermate offspring from heterozygous intercrosses was digested with *Bam*HI and hybridized with the indicated 5′ probe.

cies. They also show no signs of impaired longevity. Since cRDH is able to catalyze *cis*-retinol oxidation, a first step needed for 9-*cis*-retinoic acid formation and an essential step for formation of the visual chromophore 11-*cis*-retinal, it seemed possible that the absence of this enzyme might alter retinoid homeostasis in the body of cRDH$^{-/-}$ mice. To explore this possibility, we measured retinoid levels in several tissues that normally express relatively high levels of cRDH (liver, testis, and kidney) and several that normally express little or no cRDH (lung and spleen). The results from these measures are given in **Table 1**. As can be seen from Table 1, serum retinol and liver, testis, and kidney total retinol levels are not statistically different for cRDH$^{+/+}$ and cRDH$^{-/-}$ mice. The total retinol levels in lung and spleen for cRDH$^{-/-}$ and cRDH$^{+/+}$ mice are statistically different but these differences are probably not physiologically meaningful.

### In situ hybridization analysis of cRDH expression in the eyes of wild-type and cRDH$^{-/-}$ mice

In situ hybridization was carried out on sections of eyes from both cRDH$^{+/+}$ and cRDH$^{-/-}$ mice. Surprisingly, hybridization of sections from cRDH$^{+/+}$ mice with a digoxigenin-labeled probe generated from full-length cRDH cDNA revealed cRDH expression in several cell types in the neural retina: ganglion cells, amacrine cells, and horizontal cells, and rod inner segments of the rod photoreceptor cells (**Fig. 2A**, **B**), in addition to that previously reported for RPE (19). Amacrine and horizontal cells were identified based on their relative positions within the inner nuclear layer. The staining that spans the inner nuclear layer between the amacrine cells and the horizontal cells may represent expression in the processes of Müller cells. Hybridization of wild-type sections with a sense probe revealed only light background staining in the cell types mentioned above (Fig. 2C). Hybridization of sections of eyes from cRDH$^{-/-}$ mice with a truncated probe that is complementary only to the deleted region of cRDH, likewise revealed no significant staining (Fig. 2D). Finally, hematoxylin staining of eye sections from cRDH$^{+/+}$ and cRDH$^{-/-}$ mice revealed no obvious alterations in the architecture and cellular composition of the retina in mutant mice (Fig. 2E and 2F).

TABLE 1. Serum retinol and tissue total retinol levels for cRDH$^{+/+}$ and cRDH$^{-/-}$ mice[a]

| | cRDH$^{-/-}$ | cRDH$^{+/+}$ |
|---|---|---|
| | $\mu M$ | |
| Serum | 0.91 ± 0.23 | 0.96 ± 0.40 |
| Liver | 58.5 ± 8.2 | 74.1 ± 13.5 |
| Kidney | 0.37 ± 0.06 | 0.38 ± 0.06 |
| Testis | 0.17 ± 0.07 | 0.12 ± 0.21 |
| Lung[b] | 6.0 ± 1.8 | 8.4 ± 0.9 |
| Spleen[c] | 0.51 ± 0.09 | 0.66 ± 0.05 |

[a] All values are given as the mean ± SD. For measurement of serum retinol concentrations 14 cRDH$^{-/-}$ and 14 cRDH$^{+/+}$ mice were employed. For all other tissue measures, five cRDH$^{-/-}$ and five cRDH$^{+/+}$ mice were employed.

[b] Statistical analysis by *t*-test indicates that the levels differ significantly ($P = 0.03$).

[c] Statistical analysis by *t*-test indicates that the levels differ significantly ($P = 0.01$).

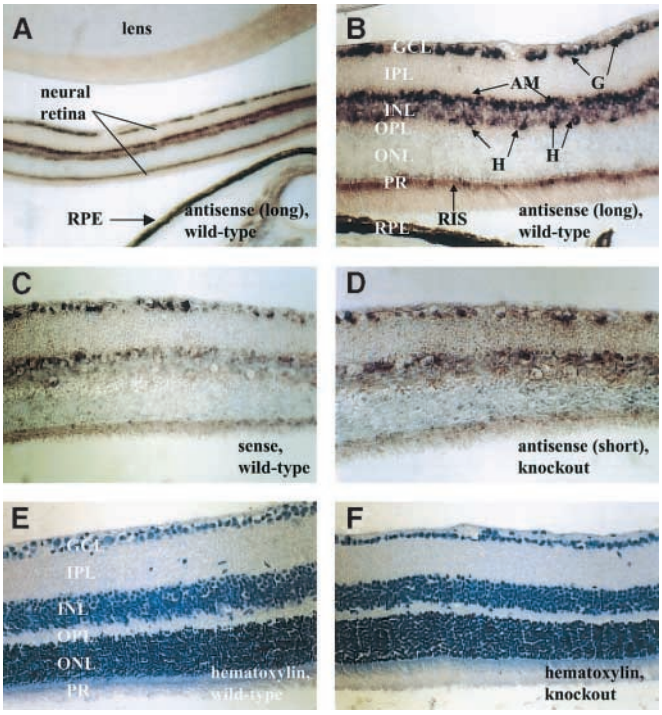


**Fig. 2.** Expression of cRDH mRNA in the retina determined by non-radioactive in situ hybridization using a full-length (long) cRDH probe. A: Low magnification (10×) view of cRDH expression in the eye. Expression can be seen in four discrete areas in the neural retina. B: High magnification (20×) view of cRDH expression in the neural retina. Strong expression is observed in most, if not all, of the ganglion cells (G), in the ganglion cell layer (GCL), in the amacrine cells (AM), and horizontal cells (H) of the inner nuclear layer (INL), and in the rod inner segments (RIS) of the photoreceptor layer (PR). Staining in the INL between the amacrine and horizontal cells may represent expression in the processes of the Müller cells. C: No expression is observed with a sense (control) probe. D: No expression is observed with the truncated probe (see Materials and Methods) that is complementary to deleted sequences. E: Hematoxylin staining of the retina from a wild-type mouse. F: Hematoxylin staining of the retina from a knockout mouse.

### Eyecup retinoid levels for dark adapted, bleached, and recovering eyes

Although cRDH is present in many tissues throughout the body, it was first identified as an eye-specific enzyme that was proposed to be essential in the visual cycle for catalyzing the oxidation of 11-*cis*-retinol to the visual chromophore 11-*cis*-retinal. Because cRDH had been proposed to play a unique and consequently essential role in maintaining vision, we investigated how the absence of this enzyme in cRDH$^{-/-}$ mice influenced retinoid levels in dark adapted, bleached, and recovering eyes. As can be seen from **Table 2**, there are no differences in the levels of all-*trans*- and 11-*cis*-retinal in dark-adapted eyes from cRDH$^{+/+}$ and cRDH$^{-/-}$ mice. However, for dark-adapted mice, total 11/13-*cis*-retinol levels (11/13-*cis*-retinol + 11/13-*cis*-retinyl ester) were elevated in the cRDH$^{-/-}$ mice as compared with control cRDH$^{+/+}$ mice (Table 2). Differ-

TABLE 2. Eye retinal and total retinol levels for dark adapted cRDH$^{-/-}$ and cRDH$^{+/+}$ mice[a]

| | cRDH$^{-/-}$ | cRDH$^{+/+}$ |
|---|---|---|
| | *ng/pair of eye cups* | |
| 11-*cis*-retinal | 192 ± 18 | 200 ± 32 |
| all-*trans*-retinal | 20 ± 6 | 18 ± 4 |
| 11/13-*cis*-retinol[b] | 64 ± 24 | 13 ± 5 |
| all-*trans*-retinol[b] | 28 ± 12 | 69 ± 25 |

[a] Values are given as the mean ± SD for measures from 10 pairs of eyes for retinal determinations and for 8 pairs of eyes for total retinol determinations. The mice were dark-adapted overnight prior to sacrifice for retinoid determinations.

[b] Statistical analysis by *t*-test indicates that these values are statistically different ($P < 0.05$) for cRDH$^{-/-}$ and cRDH$^{+/+}$ mice.

ences in eye cup retinoid levels also were observed when the eyes of cRDH$^{-/-}$ mice underwent a strong bleach and were allowed to recover. As is seen in **Fig. 3**, 11-*cis*-retinal was regenerated much more slowly in cRDH$^{-/-}$ mice during recovery from bleaching than in cRDH$^{+/+}$ mice. Moreover, the cRDH$^{-/-}$ mice accumulated much greater levels of 11/13-*cis*-retinyl esters during recovery from photobleaching than was observed for wild-type mice (Fig. 3). It is clear from these data that the absence of cRDH has a marked effect on how rapidly 11-*cis*-retinal is regenerated.

### ERG recordings

Since 11-*cis*-retinal regenerated at a much slower rate in cRDH$^{-/-}$ mice than cRDH$^{+/+}$ mice, we investigated whether this biochemical difference was also manifested in the ERG responses of these mice to light. We observed that the maximum amplitudes and the relationship between light intensity and amplitude of stimulation of the dark-adapted ERGs of the cRDH$^{-/-}$ and cRDH$^{+/-}$ mice were not significantly different from that of the cRDH$^{+/+}$ mice. This indicates that the mutant mice do not have a retinal degeneration arising from the absence of cRDH. Recovery from light adaptation, however, did show a difference between cRDH$^{+/+}$ and cRDH$^{-/-}$ mice. **Figure 4** shows examples of the recovery of the ERG after 5 min of light adaptation. In the dark-adapted state, there are large a- and b-waves with oscillations on the rising phase of the b-wave. Within the first few minutes after light adaptation, the a-wave is absent and the b-wave is reduced in amplitude. Gradually these responses return to the dark-adapted levels. **Figure 5** shows that the a-wave amplitude of the cRDH$^{-/-}$ mice recovers more slowly than cRDH$^{+/-}$ or cRDH$^{+/+}$ mice from this relatively strong adapting light. This difference is most pronounced during the later stage of adaptation.

## DISCUSSION

cRDH$^{-/-}$ mice generated by us and by others are generally normal, displaying only a subtle visual phenotype (19). This is somewhat surprising considering that cRDH has been proposed to be involved in the synthesis of 9-*cis*-

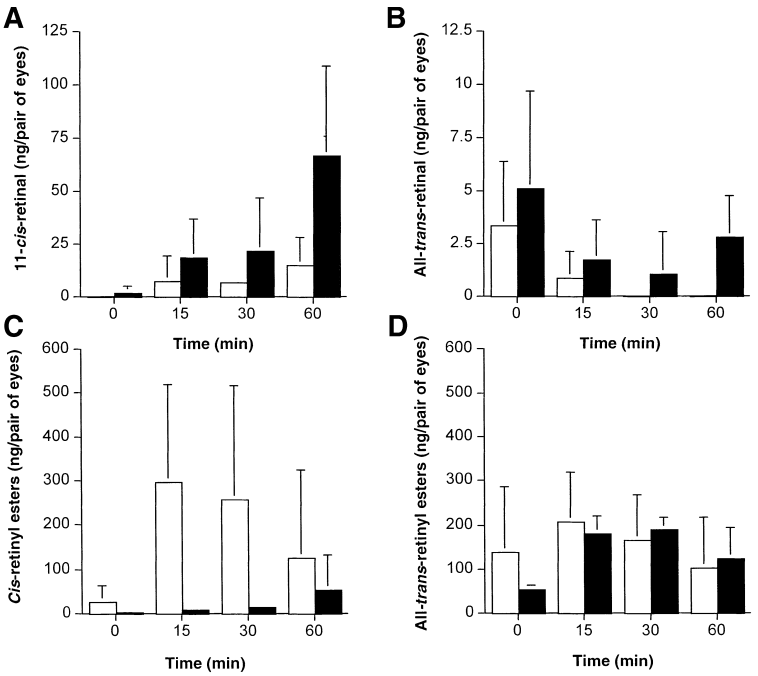


**Fig. 3**. A, B: Concentrations of 11-*cis*- and all-*trans*-retinal in the eyes of dark-adapted cRDH$^{+/+}$ (dark bars) and cRDH$^{-/-}$ (open bars) mice 0, 15, 30, and 60 min after an intense photobleach. Both 11-*cis*-retinal and all-*trans*-retinal levels for cRDH$^{-/-}$ and cRDH$^{+/+}$ mice were statistically different ($P < 0.01$) at 60 min. For the other times, statistically significant differences in retinal levels between cRDH$^{-/-}$ and cRDH$^{+/+}$ mice were not observed. C, D: Concentrations of 11-*cis*-/13-*cis*-retinyl ester and all-*trans*-retinyl ester in the eyes of dark-adapted cRDH$^{+/+}$ (dark bars) and cRDH$^{-/-}$ (open bars) mice 0, 15, 30, and 60 min after bleaching. No statistically significant differences between cRDH$^{-/-}$ and cRDH$^{+/+}$ mice for either *cis*- or all-*trans*-retinyl ester levels were observed at 0, 15, 30, or 60 min. The error bars indicate one SD.

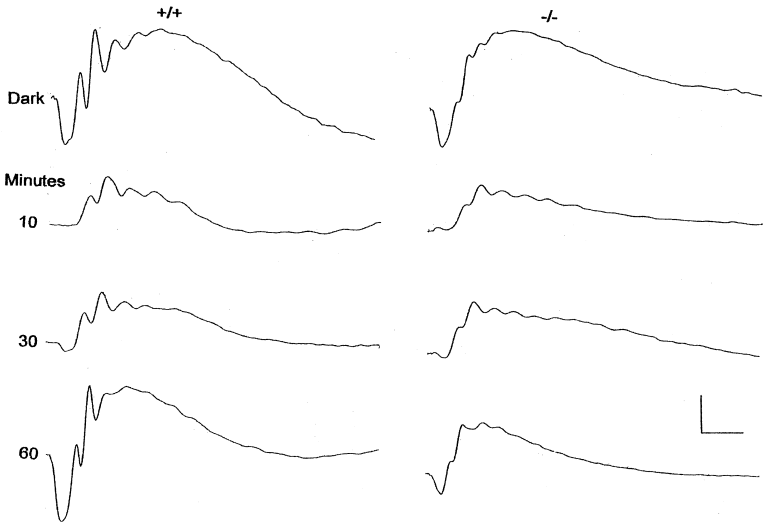


**Fig. 4**. Electroretinagrams from cRDH$^{+/+}$ (+/+) and cRDH$^{-/-}$ (−/−) mice in the dark-adapted state and at different times during recovery from light adaptation. The calibration at the lower right indicates 200 μV vertically and 30 ms horizontally.

retinoic acid from 9-*cis*-retinol (1, 7–9), to be important for catalyzing formation of active androgen forms (9, 10) and to play an essential role in the visual cycle (6). The existence of the cRDH$^{-/-}$ mice suggests that none of these biochemical functions is essential and that, at best, each proposed physiologic action of cRDH is redundant.

Although cRDH was the first enzyme identified that was proposed to be involved in the pathway leading to the formation of 9-*cis*-retinoic acid from 9-*cis*-retinol (1), several other members of the short chain dehydrogenase-reductase protein family have since been identified as being able to catalyze 9-*cis*-retinol oxidation (20, 21). In addition, several members of the medium chain alcohol dehydrogenase protein family have now been shown to catalyze this first oxidative step toward 9-*cis*-retinoic acid formation (21). Nevertheless, cRDH is highly expressed early in mouse embryogenesis by embryonic day 6.5, and is the earliest of any enzyme proposed to be involved in retinoic acid synthesis (22). Since the cRDH$^{-/-}$ mice show no deficits of the types that have been observed during embryogenesis of the various retinoid X receptor (RXR)-knockout mouse strains, this would seem to rule out the possibility that the absence of this enzyme can lead to insufficient 9-*cis*-retinoic acid synthesis within the embryo and consequently, embryonic malformations. Since 9-*cis*-retinoic acid levels are very low in rodent tissues (<1 ng/g tissue or <3.3 nM) (23), we were not able to determine by HPLC techniques whether tissue 9-*cis*-retinoic acid levels for cRDH$^{-/-}$ mice were indeed lower than those found in cRDH$^{+/+}$ mice. However, as seen from Table 1, there are no gross differences in tissue total retinol levels for the cRDH$^{-/-}$ mice compared with cRDH$^{+/+}$ mice. These observations are consistent with the conclusion that cRDH is not the sole enzyme involved in 9-*cis*-retinoic acid synthesis within either the embryo or the adult organism and that absence of cRDH does not significantly impair the retinoid needs of the organism.

In vitro studies indicate that cRDH can catalyze the interconversion of 5α-androstane-3α,17β-diol and dihydrotestosterone, and that of androsterone and androstanedione (9, 10). These reactions could constitute a nonclassical pathway for the production of active androgens in peripheral tissues. Moreover, it is known that cRDH is expressed in a variety of reproductive tissues including testis, uterus, and ovary (1). However, our investigations of cRDH-deficient mice do not suggest that the enzyme is acting in an essential manner in catalyzing active androgen production. Both male and female cRDH$^{-/-}$ mice are fertile, showing normal sexual development and no overt abnormalities in the reproductive system have been observed. We have not observed differences in fertility rates for cRDH$^{-/-}$ compared with cRDH$^{+/+}$ mice. Considering the lack of a phenotype in cRDH$^{-/-}$ mice, it would appear that the synthesis of androgens is not abnormal in these mice and consequently cRDH does not act in vivo in an essential manner for catalyzing active androgen production.

The cRDH$^{-/-}$ mice do display a mild visual phenotype. ERGs from cRDH$^{-/-}$ mice after full dark adaptation are comparable to cRDH$^{+/+}$ mice as is their dark adaptation after short bleaching. However, the regeneration of rhodopsin after prolonged bleaching in knockout mice is much slower than wild-type mice. The delay in rhodopsin formation is accompanied by an elevation in total *cis*-retinol levels (11/13-*cis*-retinol + 11/13-*cis*-retinyl ester) in the eyes of the cRDH$^{-/-}$ mice, suggesting that the conversion of 11-*cis*-retinol to 11-*cis*-retinal is at least partially blocked or greatly slowed. In humans, mutation of the cRDH gene results only in a relatively mild phenotype, *fundus albipunctatus*, a kind of stationary night blindness (24). In vitro expression studies of the mutant form of human cRDH showed that the mutant enzyme has no activity (24, 25). The most straightforward explanation for these results is that there is a redundant enzyme able to catalyze 11-*cis*-

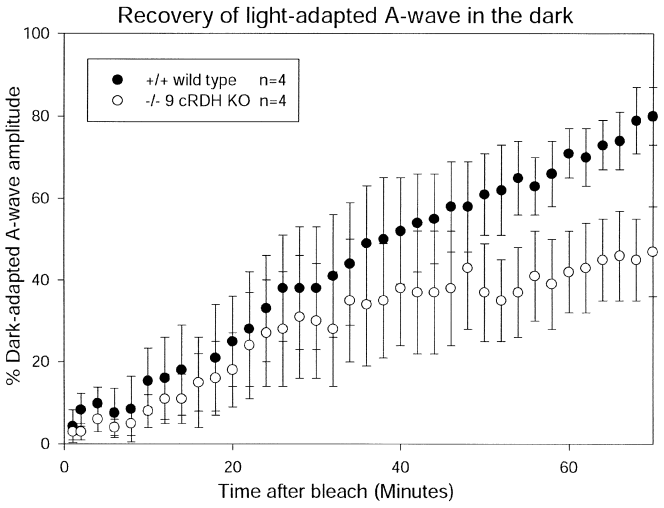


**Fig. 5.** Recovery of a-wave amplitude of cRDH$^{+/+}$ (closed circle) and cRDH$^{-/-}$ (open circle) mice after exposure to an adapting field. The vertical line at each data point is the standard error of the mean.

retinol oxidation in the human RPE. Indeed, Saari and coworkers (26) and Cideciyan et al. (25) did demonstrate a NADP$^+$/NADPH-dependent red-ox activity that can oxidize 11-*cis*-retinol in bovine RPE microsome preparations (25, 26). The NADP$^+$/NADPH-dependent red-ox activity in the bovine RPE was found by Cideciyan et al. (25) to work at low efficiency compared with bovine cRDH, which is NAD$^+$/NADH dependent. Considering the relatively mild phenotype of the cRDH$^{-/-}$ mice, it seems probable that like the human and bovine RPE, the mouse RPE also possesses an enzymatic activity that in the absence of cRDH is able to catalyze the oxidation of 11-*cis*-retinol.

Surprisingly, we observed expression of cRDH in the neural retina. In particular, robust expression was observed in the ganglion, amacrine, and horizontal cells, as well as in rod inner segments. The horizontal, amacrine, and ganglion cells are all involved in the neuronal relay that transfers visual signals from the photoreceptor layer to the optic nerve (27). A number of findings suggest that there is indeed active retinoid metabolism in the eye apart from that in the photoreceptor-RPE visual cycle. For example, cellular retinoic acid-binding protein (CRABP) has been immunolocalized to amacrine and bipolar cells of the chick retina (28). Moreover, retinaldehyde dehydrogenase type 2 (RALDH2), retinoic acid receptors, and RXRs have been found in amacrine cells, while RXRs have also been observed in ganglion cells, and cellular retinaldehyde-binding protein (CRALBP) in Müller cells (28, 29). Our data complement these previous findings that CRABP and CRALBP and enzymes involved in retinoid transformations are present in the neural retina cells other than photoreceptors and the RPE.

We suggest that there are at least three possible roles for cRDH and retinoid signaling in the neural retina. First, the finding that the rod and cone photoreceptors are not required for maintenance of circadian rhythms in mammals (30) suggests that photoentrainment might be mediated by as yet unidentified photoreceptors in the neural retina. The presence of a novel opsin, melanopsin, in the ganglion and amacrine cells of the rodent and primate retina (31), along with the discovery of blue light-absorbing cryptochromes in mouse ganglion cells (32), raises the possibility that retinoids might be involved in the entrainment of circadian rhythms in neural elements of the inner retina. However, this is argued against by recent studies of vitamin A-deficient retinol-binding protein-deficient mice that show a normal circadian clock even though these mice are totally blind (33). Second, retinoic acid has been shown to enhance neurite outgrowth of chick retinal ganglion cells in vitro (34). This result is consistent with the suggestion that retinoid signaling is involved in maintenance of neuronal phenotypes in the retina (28, 29). Finally, retinoic acid has been shown to be involved in the regulation of apoptosis in a wide array of tissues. Most relevant to this discussion, in the Mitf$^{vit}$ mouse model of retinal degeneration there is increased photoreceptor apoptosis, possibly resulting from the observed increase in retinoic acid production (35). Interestingly, these mice also exhibit an elevation of RALDH2 expression in the eye in a pattern that is strikingly similar to the normal pattern of expression observed for cRDH (35). If cRDH were required for the conversion of 9-*cis*-retinol to 9-*cis*-retinal in the neural retina, the possibility exists that the Mitf$^{vit}$ apoptotic phenotype might be less severe on the cRDH$^{-/-}$ background. These potential physiologic roles for cRDH in the neural retina can be tested using the cRDH knockout model.

Recently a publication by Jang et al. appeared and provided a description of cRDH$^{-/-}$ mice and the slowed regeneration of 11-*cis*-retinal upon prolonged bleaching of dark-adapted cRDH$^{-/-}$ mice (36). Our data are consistent with those provided in this other report.

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